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(54) Title: METHODS FOR IDENTIFYING GENES ESSENTIAL TO THE GROWTH OF AN ORGANISM

(57) Abstract

The present invention provides methods for identifying genes essential to the growth of an organism using a grid prepared from a genomic library of a selected organism. Genes identified as essential by this method and proteins encoded thereby are also provided. In addition, methods of using these genes and proteins encoded thereby are disclosed.

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METHODS FOR IDENTIFYING GENES ESSENTIAL TO THE GROWTH OF AN ORGANISM

FIELD OF THE INVENTION

The present invention relates to the use of high-density arrays or grids of genomic (or cDNA) libraries for the identification, sequencing and characterization of genes which are essential to the growth of an organism, and more specifically to a pathogen. The determination of these essential genes and the proteins encoded thereby is useful in the development of new therapies against such pathogens.

BACKGROUND OF THE INVENTION

Identification, sequencing and characterization of genes is a major goal of modern scientific research. By identifying genes, determining their sequences and characterizing their biological function, it is possible to employ recombinant technology to produce large quantities of valuable gene products, e.g. proteins and peptides. Additionally, knowledge of gene sequences can provide a key to diagnosis, prognosis and treatment in a variety of infectious diseases and disease states in plants and animals which are characterized by inappropriate expression and/or repression of selected genes or by the influence of external factors, e.g., carcinogens or teratogens, on gene function.

Methods have been described for the identification of certain novel gene sequences, referred to as Expressed Sequence Tags (EST). Adams *et al.*, *Science*, 1991, 252:1651-1656. A variety of techniques have also been described for identifying particular gene sequences on the basis of their gene products. For example, see International Patent Application No. WO91/07087, published May 30, 1991. In addition, methods have been described for the amplification of desired sequences. For example, see International Patent Application No. WO91/17271, published November 14, 1991.

Genes which are essential for the growth of an organism, however, have been difficult to identify in such a manner as to be easily recovered for future analysis. The most common methodology currently employed to identify essential genes is a multi-

step process involving the generation of a conditionally lethal mutant library followed by the screening of duplicate members under the appropriate permissive and non-permissive conditions. Candidate mutants are then transformed with a second, genomic library and the desired genes isolated by complementation of the mutant phenotype. The complementing plasmid is recovered, subcloned, and then retested. However, this procedure comprises multiple subcloning steps to identify and recover the desired genes thus making it both labor intensive and time consuming.

Accordingly, there exists a need for a more efficient method of identifying genes essential to the growth of an organism.

SUMMARY OF THE INVENTION

In one aspect, the invention provides a method of identifying a gene or genes which are essential to the growth of an organism through the use of high density arrays or grids of genomic libraries. The method involves preparing a genomic library of a selected organism and providing a plurality of identical grids, each grid comprising a surface on which is immobilized at predefined regions on said surface a plurality of defined materials derived from the genomic library. The selected organism is then mutagenized, preferably by insertional mutagenesis, and grown in a test culture under a selected set of defined conditions. A control culture comprising the non-mutagenized selected organism is also grown under the same set of defined conditions. Surviving cells from the cultures are harvested and DNA from harvested cells of the mutagenized organism (test culture) and RNA, or DNA, from harvested cells of the non-mutagenized organism (control culture) are extracted and isolated. Labeled polynucleotide probes from the isolated DNA of the test culture and labeled polynucleotide probes from the isolated RNA (or DNA) of the control culture are then generated and hybridized to identical grids to produce a test hybridization pattern and a control hybridization pattern, respectively. Hybridization patterns on the grids are then compared to identify genes essential for growth of the selected organism. Essentiality of the identified gene for growth of the selected organism is then confirmed.

The method of the present invention may further comprise growing additional test cultures comprising the mutagenized organism and control cultures comprising the non-mutagenized organism under different sets of defined conditions. Labeled probes from the isolated DNA and RNA from these additional cultures are generated in the same fashion as previously described to produce test and control hybridization patterns for cultures grown under the different sets of defined conditions. Genes essential to the growth of the selected organism are then identified by comparing the hybridization patterns generated by mutagenized and non-mutagenized organisms grown under each of the different sets of defined conditions.

An additional aspect of the invention provides an isolated gene which is essential to the growth of an organism and is identified by one of the above methods.

Yet another aspect of the invention is an isolated protein produced by expression of the gene sequence identified above. Such proteins are useful in the development of therapeutic and diagnostic compositions, or as targets for drug development.

Yet another aspect of the invention is to identify broad spectrum antibiotics or antifungals which inhibit the expression of these essential genes.

In a related aspect, the present invention provides a method to identify conditionally lethal mutant genes of a selected organism by complementation with a non-mutagenized genomic library of the same organism. The method involves preparing a genomic library in either an integration vector, or in an expression vector, and providing a grid comprising a surface on which is immobilized at predefined regions on said surface a plurality of defined materials derived from the genomic library. The selected organism is then mutagenized, preferably by chemically induced point mutations, and grown (in a test culture) under permissive and non-permissive conditions to identify mutagenized organisms that contain conditionally lethal mutant genes. Organisms that contain conditionally lethal mutant genes are transformed with the prepared (i.e., non-mutagenized) genomic library and the transformed organisms, or cells, are grown under the same non-permissive conditions used to identify mutagenized organisms that contain the conditionally lethal mutant genes. Surviving cells are harvested and DNA is extracted and isolated. Labeled polynucleotide probes

from the isolated DNA are then generated and hybridized to the grid to identify genes essential for growth of the selected organism.

Other objects, features, advantages and aspects of the present invention will become apparent to those of skill in the art from the following description. It should be understood, however, that the following description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only. Various changes and modifications within the spirit and scope of the disclosed invention will become readily apparent to those skilled in the art from reading the following description and from reading the other parts of the present disclosure.

DETAILED DESCRIPTION OF THE INVENTION

The biochemical basis of many bacterial resistance mechanisms to antibiotics is now known. These mechanisms alone, or in concert, are responsible for the escalating problem of antibiotic resistance seen both in hospital and community acquired infection. The principle approach by researchers to overcome these problems has been to seek incremental improvements in existing drugs. Although these approaches contribute somewhat to the fight against infection by such resistant pathogens, new approaches are needed.

Methods have now been developed for identifying genes and gene products essential to the survival of an organism. Genes and gene products identified by these methods are useful as molecular targets for drug discovery. The methods of the present invention are useful in determining the effect of the total absence of a gene or gene product on the survival of an organism.

I. Definitions

Several words and phrases used throughout this specification are defined as follows:

As used herein, the term "gene" refers to the genomic nucleotide sequence from which a cDNA sequence is derived. The term gene classically refers to the genomic sequence, which upon processing, can produce different cDNAs, e.g., by

splicing events. However, for ease of reading, any full-length counterpart cDNA sequence will also be referred to by shorthand herein as gene.

By "gene product" it is meant any polypeptide sequence encoded by a gene. The term "genomic library" is meant to include, but is not limited to, plasmid libraries, PCR products from genomic libraries, cDNA libraries and known sequences. Methods for the construction of such libraries are well known by those skilled in the art. In a preferred embodiment of the present invention, a genomic library is constructed in a suicide vector. It is also preferred that the constructed library be adjusted to minimize the number of complete genes present in a single genomic insert to approximately one gene. Techniques for this adjustment are well known to the skilled artisan.

"Isolated" means altered "by the hand of man" from its natural state; i.e., that, if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a naturally occurring polynucleotide or a polypeptide naturally present in a living animal in its natural state is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. For example, with respect to polynucleotides, the term isolated means that it is separated from the chromosome and cell in which it naturally occurs.

By "organism" it is meant any single cell organism. Preferably this includes, but is not limited to, bacterium (including both gram negative and gram positive species), viruses and lower eukaryotic cells such as fungi, yeast, molds and simple multicellular organisms. Preferably, the organism is a pathogen.

The term "pathogen" is defined herein as any organism which is capable of infecting an animal or plant and replicating its nucleic acid sequences in the cells or tissue of that animal or plant. Such a pathogen is generally associated with a disease condition in the infected animal or plant. Such pathogens may include, but are not limited to, viruses, which replicate intra- or extra-cellularly, or other organisms such as bacteria, fungi or molds, which generally infect tissues or the blood. Certain pathogens are known to exist in sequential and distinguishable stages of development, e.g., latent stages, infective stages, and stages which cause symptomatic diseases. In

these different states, the pathogen is anticipated to rely upon different genes as essential for survival or for pathogenicity.

As used herein, the term "solid support" refers to any known substrate which is useful for the immobilization of a plurality of defined materials derived from a genomic library by any available method to enable detectable hybridization of the immobilized polynucleotide sequences with other polynucleotides in the sample. Among a number of available solid supports, one desirable example is the supports described in International Patent Application No. WO91/07087, published May 30, 1991. Examples of other useful supports include, but are not limited to, nitrocellulose, nylon, glass, silica and Pall BIODYNE C. It is also anticipated that improvements yet to be made to conventional solid supports may also be employed in this invention.

The term "grid" means any generally two-dimensional structure on a solid support to which the defined materials of a genomic library are attached or immobilized.

As used herein, the term "predefined region" refers to a localized area on a surface of a solid support on which is immobilized one or multiple copies of a particular clone and which enables hybridization of that clone at the position, if hybridization of that clone to a sample polynucleotide occurs.

By "immobilized", it is meant to refer to the attachment of the genes to the solid support. Means of immobilization are known and conventional to those of skill in the art, and may depend on the type of support being used.

II. Compositions of the Invention

The present invention is based upon the use of high density arrays or grids of genomic libraries as a means for rapidly identifying genes essential for the growth of an organism.

A. Preparation of genomic libraries

For this analysis a random genomic library for the target organism is prepared. The genomic DNA is isolated using standard procedures for molecular biology such as

those disclosed by Sambrook *et al.*, MOLECULAR CLONING, A LABORATORY MANUAL, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989. The genomic library is then constructed in accordance with procedures described by Fleischmann et al. *Science*, 1995, *269*:496-512. For the purposes of the present invention, a genomic library can comprise a plasmid library, PCR products from a genomic library, or known sequences. In one embodiment, a suicide vector is used for preparation of the genomic library. Examples of suicide vectors which may be used in the present invention are well known in the art. See, for example, Booker *et al. Lett. Appl. Microbiol.* 1995 *21*:292-297; Steinmeitz, M. and Richter, R. *Gene*, 1994, *142*:79-83; Yu et al. *J. Bacteriol.* 1994 *176*:3627-34; and Quandt, J. and Hynes, M.F. *Gene*, 1993, *127*:15-21. In a preferred embodiment, a suicide vector containing the broad host range erythromycin (Erm) gene can be prepared in a commercially available plasmid such as pBluescript (pBS; Stratagene, La Jolla, CA). The Erm gene is isolated as a Taq1 restriction fragment from the vector pE194 (Hourinouchi, S. and Weisbaum, B. J. Bacteriology 1982, 150:804-812). The Erm containing fragment is ligated directly into NaeI digested, CIP-treated pBS and transformed into HB101 cells. Transformants are screened by PCR to determine the presence of the Erm gene. Using this vector, two Erm positive isolates were confirmed by sequence analysis and designated pJMErmA4 and pJMErmD2. For library construction, genomic inserts are placed into the unique SmaI site present in the polylinker region. It is also preferred that the constructed library be adjusted to minimize the number of complete genes present in a single genomic insert. Techniques for making this adjustment to the library are well known to those skilled in the art.

B. Preparation of Grid

A plurality of materials derived from the genomic library are gridded onto a surface of a solid support at predefined locations or regions, preferably at 6X coverage. By "plurality of materials derived from the genomic library" it is meant to include, but is not limited to, bacterium containing individual clones spotted onto and grown on a surface of the solid support at predefined locations or regions; or plasmid clones isolated from said library, PCR products derived from the inserts from the

plasmid clones, or oligonucleotides derived from sequencing of the plasmid clones, which are immobilized to the surface of the solid support at predefined locations or regions.

Numerous conventional methods are employed for immobilizing these materials to surfaces of a variety of solid supports. See, e.g., Affinity Techniques, Enzyme Purification: Part P, Methods in Enzymology, Vol. 34, ed. W.B. Jakoby, M. Wilcheck, Acad. Press, NY (1971); Immobilized Biochemicals and Affinity Chromatography, Advances in Experimental Medicine and Biology, Vol. 42, ed. R. Dunlap, Plenum Press, NY (1974); U.S. Patent 4,762,881; U.S. Patent No. 4,542,102; European Patent Publication No. 391,608 (October 10, 1990); or U.S. Patent No. 4,992,127 (November 21, 1989).

One desirable method for attaching these materials to a solid support is described in International Application No. PCT/US90/06607 (published May 30, 1991). Briefly, this method involves forming predefined regions on a surface of a solid support, where the predefined regions are capable of immobilizing the materials. The method makes use of binding substrates attached to the surface which enable selective activation of the predefined regions. Upon activation, these binding substances become capable of binding and immobilizing the materials derived from the genomic library.

Any of the known solid substrates suitable for binding nucleotide sequences at predefined regions on the surface thereof for hybridization and methods for attaching nucleotide sequences thereto may be employed by one of skill in the art according to the invention. Similarly, known conventional methods for making hybridization of the immobilized materials detectable, e.g., fluorescence, radioactivity, photoactivation, biotinylation, energy transfer, solid state circuitry, and the like may be used in this invention.

C. Preparation and Growth of Mutagenized Organism

The organism of interest is mutagenized by transfection with either a randomly integrating transposon or similar insertional or transposable elements of known

sequence (e.g., Tn, IS, phage Mu, Ty element) or with a constructed suicide vector and allowed to grow under a selected set of defined conditions.

III. The Methods of the Invention

A. Identification of Genes

The present invention employs the compositions described above in methods for identifying genes which are essential to the growth of an organism. These methods may be employed to detect such genes, regardless of the state of knowledge about the function of the gene.

In one embodiment, a gene or genes which are essential to the growth of a selected organism are identified through the use of two or more identical high density arrays or grids of genomic libraries prepared from the selected organism. For this analysis, at least two identical high density grids or arrays are prepared. Each grid is prepared from a random genomic library for a selected organism, preferably in a suicide vector. A plurality of defined materials derived from the genomic library are then gridded onto a solid support, preferably at 6X coverage. The insert size of this library is adjusted to minimize the number of complete genes that might be present in a single insert. In a preferred embodiment, the target insert size is one complete gene. For bacteria, the average length of a complete gene is approximately 1 kb.

The selected organism is mutagenized by transfection with either a randomly integrating transposon or similar insertional or transposable element of known sequence, such as Tn, IS, Ty element or phage Mu, or with the constructed suicide vector. The mutagenized selected organism is then cultured under a selected set of defined *in vitro* or *in vivo* conditions to produce a test culture. In addition, a non-mutagenized selected organism is also cultured under the same set of defined conditions to produce a control culture. By "defined conditions" it is meant, but is not limited to, standard *in vitro* culture conditions recognized as normal (i.e., non-pathogenic) for a selected organism, as well as *in vitro* conditions which reflect or mimic *in vivo* pathogenic settings (conditions) such as heat shock, auxotrophic, osmotic shock, antibiotic or drug selection/addition, varied carbon sources, and aerobic or anaerobic conditions, and *in vivo*, pathogenic conditions. Preferably, such

conditions are predetermined to allow maximum growth of the non-mutagenized organism. The surviving cells are then harvested. Harvesting can be performed during various growth stages of the cells to ascertain the essentiality of a particular gene during different stages of growth. For example, harvesting can be performed during early logarithmic growth, late logarithmic growth, stationary phase growth or late stationary growth. RNA (or DNA) is then extracted and isolated from the harvested non-mutagenized cells of the control culture, while DNA is extracted and isolated from the mutagenized cells of the test culture using standard methodologies well known to those skilled in the art.

RNA (or DNA) extracted from the non-mutagenized cells of the control culture and DNA extracted from the mutagenized cells of the test culture are then used to generate labeled probes. The extracted, isolated DNA of the test culture serves as templates in primer extension reactions using oligonucleotide primers directed against a transposon/integrated vector sequence and which extends into the neighboring (i.e., flanking) nucleic acid sequence of the (genomic) DNA. Such primers will vary depending upon the mutagenesis/vector system employed. For example, in one embodiment, where the libraries constructed in the pJMErmA4 or pJMErmD2 vectors are used for both gridding and mutagenesis, primers designated against sequences which flank the SmaI cloning site are used. Examples of such primers include, but are not limited to:

5'-AATTAACCCTCACTAAAGGGAACA-3' (SEQ ID NO:1);

5'-TGTTCCCTTTAGTGAGGGTTAATT-3' (SEQ ID NO:2);

5'-GTAATACGACTCACGGAGGGGCGA-3' (SEQ ID NO:3); and

5'-ACGCCCCTCCGTGAGTCGTATTAG-3' (SEQ ID NO:4).

The extension reactions are performed using detectably labeled, i.e. radio- or fluorescent dye-labeled or biotinylated, nucleotides and controlled so that the extension products average approximately 200 base pairs (bp) in length. A number of methods exist for generating the primer extension products. In one embodiment, primer extension reactions are performed under the following conditions: A sample containing 15 pmoles of appropriate primer or primers, 5 pmoles extracted DNA, 30 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 0.1 mM dATP, 0.1 mM ^{32}P-dCTP,

0.1 mM dGTP, 0.1 mM dTTP, 0.25 mM ddATP, 0.25 mM ddCTP, 0.25 mM ddGTP and water to 135 µl is prepared. This sample is then incubated at 75°C for 15 minutes; 50°C for 30 minutes and 37°C for 15 minutes. Klenow polymerase (75 units in a total volume of 15 µl) is then added and the sample is incubated for 30 minutes at 37°C. EDTA to 20 mM is the added. The sample is then extracted 1 time each with phenol, chloroform and isoamyl alcohol, followed by a second extraction with chloroform and isoamyl alcohol. The product is then precipitated with ethanol.

When RNA (or DNA) from the non-mutagenized organism is used to generate the probes, isolated RNA (or DNA) is labeled according to standard methods using random primers, preferably hexamers, and reverse transcriptase. Such methods are routinely performed by those skilled in the art.

These labeled products are then used as hybridization probes against the identical high density grids. Labeled probes prepared from DNA extracted from mutagenized cells of the test culture are hybridized to one identical grid, while labeled probes from the RNA extracted from the non-mutagenized cells of the control culture are hybridized to a second identical grid. The generated test hybridization patterns and control hybridization patterns are then compared. Genes essential for the growth of the selected organism are identified by determining differences at the predefined regions of the grids between the test hybridization pattern and the control hybridization pattern grown under the selected set of defined conditions

Alternatively, additional test cultures comprising the mutagenized selected organism and control cultures comprising the non-mutagenized selected organism are grown under different sets of defined *in vitro* and *in vivo* conditions. Hybridization patterns for labeled polynucleotide probes prepared from DNA of the additional test cultures and RNA of the additional control cultures are then generated in accordance with procedures described herein. Genes essential to the growth of the organism are then identified by comparing the hybridization patterns of the test and control cultures for each set of defined conditions with each other. In one embodiment, genes essential to the growth of the organism will be those common to all of the hybridization patterns for all the cells. In another embodiment, genes essential for

growth of a selected organism will hybridize under one set of growth conditions and will not hybridize under a different set of growth conditions.

In another embodiment, a pool of conditionally lethal mutants of the organism can be generated and transformed with a second (genomic) library constructed in a transposon/integration based vector. Transformants are reselected under the original conditionally lethal conditions and the rescued, surviving isolates used for probe generation and hybridization analysis as described above. For example, a temperature sensitive (ts) mutant library is prepared according to standard procedures and screened under permissive vs. non-permissive conditions to identify conditionally lethal mutants. The identified conditionally lethal ts mutants are pooled and transformed with a second, genomic library constructed in a transposon/integration based vector containing both a conditional and a selectable marker system. Examples of vectors for this second library include, but are not limited to, pMAK705 (Bloomfield et al. *Mol. Microbiol.* 1991 *5*:1447-1457) and pG+host5 (Biswas et al. *J. Bacteriol.* 1993, 175:3628-3635). The resulting transformants are retested or grown under the original temperature selection for lethality/essentiality. Survivors represent isolates containing integrated vector plus complementing genomic sequences. DNA from these survivors is then isolated and probes are generated as described in the preceding paragraphs, whereby hybridizing clones identify essential genes of interest.

In yet another embodiment, a conditionally lethal mutant library is prepared according to standard procedures, is constructed in an expression vector, and transformed with a selectable, genomic library. The genomic library is constructed using standard molecular biology techniques such that expression of the inserted genomic DNA is under control of vector-located promoter sequences, and preferably contains selectable and conditional markers. Examples of vectors containing inducible promoter systems include, but are not limited to, pFL10 (Lopez de Felipe et al. *FEMS Microbiol. Lett.* 1994, *122*: 289-295) and pUB110 (Zyprian, E. and Matzura, H. *DNA* 1986, *5*:219-225). In this embodiment, temperature sensitive lethal mutants are screened under temperature sensitive selection and under induction conditions for the vector-located promoter sequences. Surviving isolates represent clones where

transcription of the exogenous plasmid insert complements the mutant phenotype. Probes are generated against the plasmid inserts and hybridized against the grids.

Essentiality of the gene to the organism is confirmed by inactivating the identified gene in the selected organism, preferably using a single gene disruption procedure such as a knock out experiment, and culturing the selected organism under the same defined conditions.

Clones identified by the methods of the instant invention can be used directly for sequence analysis and for knockout experiments to confirm their essentiality to the growth of the organism. Alternatively, a gene sequence from the identified clone can be subcloned into a suitable vector for knockout experiments as is common in the art. Sequence analysis is performed using standard methodologies well known to those skilled in the art. Initial sequencing may be performed using the M13 universal forward and universal reverse sequencing primers which flank the multiple cloning site of the vector. The resulting sequences are analyzed using conventional computer programs. Results of said analysis are used in determining the potential usefulness of the individual clones as antimicrobial targets.

For knockout experiments, plasmid DNA from the identified isolates is purified and transformed in a non-mutagenized organism using standard molecular biology techniques. The transformed cells are grown under antibiotic selection for the vector sequence. Surviving cells represent site-specific insertional events into genes which are not essential for growth since knockout of an essential gene would result in no viable transformants. DNA is isolated from the surviving cells and used as a template to generate probes in accordance with previously described procedures and the grids reprobed for analysis. Additional gene knockout experiments can be performed in accordance with procedures described by, for example, Guiterrez et al. *J. Bacteriol.* 1996 *178*:4166-4175. Gene knockout experiments thus provide information on the effect of the total absence of the gene product.

B. Other Methods of the Invention

As is obvious to one of skill in the art upon reading this disclosure, the compositions and methods of the invention may also be used for other similar

purposes. For example, in one embodiment, this method can be used to monitor the effect of potential drugs on essential gene expression, both in laboratories and during clinical trials with animals, especially humans. Because the method can be readily adapted by altering growth conditions or the stage at which the cells are harvested, it can essentially be employed to identify essential genes of any organism, at any stage of development, and under the influence of any factor which can affect gene expression.

IV. The Genes and Proteins Identified

Application of the compositions and methods of this invention as above described also provides other compositions, such as any isolated gene sequence which is essential to the growth of an organism. Another embodiment of this invention is any isolated pathogen gene sequence found to be essential to the survival of the pathogen in a host. Similarly, an embodiment of the invention is any gene sequence identified by the methods described therein.

These gene sequences may be employed in conventional methods to produce isolated proteins encoded thereby. To produce a protein of this invention, the DNA sequences of a desired gene invention or portions thereof identified by use of the methods of this invention are inserted into a suitable expression system. In a preferred embodiment, a recombinant molecule or vector is constructed in which the polynucleotide sequence encoding the protein is operably linked to a heterologous expression control sequence permitting expression of the human protein. Numerous types of appropriate expression vectors and host cell systems are known in the art for mammalian (including human), insect, yeast, fungal and bacterial expression.

The transfection of these vectors into appropriate host cells, whether mammalian, bacterial, fungal or insect, or into appropriate viruses, results in expression of the selected proteins. Suitable host cells, cell lines for transfection and viruses, as well as methods for construction and transfection of such host cells and viruses are well-known. Suitable methods for transfection, culture, amplification, screening and product production and purification are also known in the art.

In one embodiment, the essential genes and proteins encoded thereby which have been identified by this invention can be employed as diagnostic compositions

useful in the diagnosis of a disease or infection by conventional diagnostic assays. For example, a diagnostic reagent can be developed which detectably targets a gene sequence or protein of this invention in a biological sample of an animal. Such a reagent may be a complementary nucleotide sequence, an antibody (monoclonal, recombinant or polyclonal), or a chemically derived agonist or antagonist. Alternatively, the essential genes of this invention and proteins encoded thereby, fragments of the same, or complementary sequences thereto, may themselves be used as diagnostic reagents. These reagents may optionally be detectably labeled, for example, with a radioisotope or colorimetric enzyme. Selection of an appropriate diagnostic assay format and detection system is within the skill of the art and may readily be chosen without requiring additional explanation by resort to the wealth of art in the diagnostic area.

Additionally, genes and proteins identified according to this invention may be used therapeutically. For example, genes identified as essential in accordance with this method and proteins encoded thereby may serve as targets for the screening and development of natural or synthetic chemical compounds which have utility as therapeutic drugs for the treatment of disease states associated with the organism. As an example, a compound capable of binding to a protein encoded by an essential gene thus preventing its biological activity may be useful as a drug component preventing diseases or disorders resulting from the growth of a particular organism. Alternatively, compounds which inhibit expression of an essential gene are also believed to be useful therapeutically. In addition, compounds which enhance the expression of genes essential to the growth of an organism may also be used to promote the growth of a particular organism.

Conventional assays and techniques may be used for screening and development of such drugs. For example, a method for identifying compounds which specifically bind to or inhibit proteins encoded by these gene sequences can include simply the steps of contacting a selected protein or gene product with a test compound to permit binding of the test compound to the protein; and determining the amount of test compound, if any, which is bound to the protein. Such a method may involve the incubation of the test compound and the protein immobilized on a solid

support. Still other conventional methods of drug screening can involve employing a suitable computer program to determine compounds having similar or complementary structure to that of the gene product or portions thereof and screening those compounds for competitive binding to the protein. Identified compounds may be incorporated into an appropriate therapeutic formulation, alone or in combination with other active ingredients. Methods of formulating therapeutic compositions, as well as suitable pharmaceutical carriers, and the like are well known to those of skill in the art.

Accordingly, through use of such methods, the present invention is believed to provide compounds capable of interacting with these genes, or encoded proteins or fragments thereof, and either enhancing or decreasing the biological activity, as desired. Thus, these compounds are also encompassed by this invention.

Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention are believed to be encompassed in the scope of the claims appended hereto.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: SMITHKLINE BEECHAM CORPORATION

(ii) TITLE OF THE INVENTION: Methods for Identifying Genes Essential to the Growth of an Organism

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: SmithKline Beecham Corporation
(B) STREET: 709 Swedeland Road
(C) CITY: King of Prussia
(D) STATE: PA
(E) COUNTRY: USA
(F) ZIP: 19046

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: Unknown
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/030,159
(B) FILING DATE: 06-NOV-1996

(viii) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 38,891
(C) REFERENCE/DOCKET NUMBER: P50572

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(B) TELEFAX: 610-270-5090
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AATTAACCCT CACTAAAGGG AACA 24

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGTTCCCTTT AGTGAGGGTT AATT 24

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTAATACGAC TCACGGAGGG GCGA 24

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACGCCCCTCC GTGAGTCGTA TTAG 24

WHAT IS CLAIMED IS:

1. A method of identifying genes essential to growth of a selected organism comprising:

(a) preparing a genomic library of a selected organism;

(b) providing a plurality of identical grids, each grid comprising a surface on which is immobilized at predefined regions on said surface a plurality of defined materials derived from the genomic library;

(c) mutagenizing the selected organism;

(d) growing a test culture comprising mutagenized selected organism and a control culture comprising non-mutagenized selected organism under a set of defined conditions;

(e) harvesting surviving cells from the cultures;

(f) extracting and isolating DNA from harvested cells of the test culture;

(g) extracting and isolating RNA or DNA from harvested cells of the control culture;

(h) generating labeled polynucleotide probes from the isolated DNA of the test culture;

(i) generating labeled polynucleotide probes from the isolated RNA or DNA of the control culture;

(j) hybridizing the labeled probes generated from the isolated DNA of the test culture to a first identical grid to produce a test hybridization pattern;

(k) hybridizing the labeled probes generated from the isolated RNA or DNA of the control culture to a second identical grid to produce a control hybridization pattern;

(l) comparing the hybridization patterns to identify genes essential for growth of the selected organism; and

(m) confirming that said identified gene is essential for growth of the selected organism.

2. The method of claim 1 wherein essential genes are identified in step (l) by determining differences between the test hybridization pattern and the control hybridization pattern.

3. The method of claim 1 wherein the set of defined conditions of step (d) comprises standard non-pathogenic *in vitro* culture conditions for the selected organism.

4. The method of claim 1 wherein the set of defined conditions of step (d) comprises *in vitro* conditions which reflect or mimic *in vivo*, pathogenic settings such as aerobic or anaerobic conditions, auxotrophic, heat-shock, osmotic-shock, addition or presence of antibiotics or drugs, carbon source variations, and *in vivo* pathogenic conditions.

5. The method of claim 1 wherein the harvesting of surviving cells of step (e) is performed during early logarithmic growth.

6. The method of claim 1 wherein the harvesting of surviving cells of step (e) is performed during late logarithmic growth.

7. The method of claim 1 wherein the harvesting of surviving cells of step (e) is performed during stationary phase growth.

8. The method of claim 1 wherein the harvesting of surviving cells of step (e) is performed during late stationary phase growth.

9. The method of claim 1 wherein:

step (d) further comprises growing additional test and control cultures under a different set of defined conditions; and

step (l) comprises comparing test and control hybridization patterns from the cells grown under the different sets of defined conditions.

10. The method of claim 9 wherein genes essential to the selected organism are identified by determining identical hybridization patterns for all of the cells grown under the different sets of defined conditions.

11. The method of claim 9 wherein genes essential to the selected organism are identified by determining differences between the test and control hybridization patterns for cells grown under the different sets of defined conditions.

12. A method of identifying genes essential to growth of a selected organism by identifying conditionally lethal mutant genes, which comprises:

(a) preparing a genomic library of a selected organism: (*i*) in an integration vector; or (*ii*) in an expression vector;

(b) providing a grid comprising a surface on which is immobilized at predefined regions on said surface a plurality of defined materials derived from the genomic library;

(c) mutagenizing the selected organism;

(d) growing the mutagenized organism under permissive and non-permissive conditions to identify mutagenized organisms containing conditionally lethal mutant genes;

(e) transforming such organisms containing said conditionally lethal mutant genes with the genomic library of step (a);

(f) growing the transformed cells under the same non-permissive conditions as step (d) to identify transformed cells in which conditionally lethal mutant genes have been complemented;

(g) harvesting surviving cells;

(h) extracting and isolating DNA from the harvested cells;

(i) generating labeled polynucleotide probes from the isolated DNA;

(j) hybridizing the labeled probes generated from the isolated DNA to a grid, whereby such probes that hybridize to the grid identify genes essential for growth of the selected organism.

13. An isolated gene sequence which is essential to growth of a selected organism which is identified by the method of claim 1.

14. An isolated protein produced by expression of a gene sequence of claim 13.

15. A therapeutic compound capable of modulating expression of the gene sequence of claim 13 for use in the treatment of a disease associated with growth of an organism.

16. A therapeutic compound capable of modulating activity of a protein of claim 14 for use in the treatment of a disease associated with growth of an organism.

17. A diagnostic composition useful for the diagnosis of a disease or infection comprising a reagent capable of detectably targeting a gene sequence of claim 13.

18. An isolated gene sequence which is essential to growth of a selected organism which is identified by the method of claim 12.

19. An isolated protein produced by expression of a gene sequence of claim 18.

20. A therapeutic compound capable of modulating expression of the gene sequence of claim 18 for use in the treatment of a disease associated with growth of an organism.

21. A therapeutic compound capable of modulating activity of a protein of claim 19 for use in the treatment of a disease associated with growth of an organism.

22. A diagnostic composition useful for the diagnosis of a disease or infection comprising a reagent capable of detectably targeting a gene sequence of claim 18.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/20004

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C07H 21/04; A61K 48/00

US CL : 436/6; 536/23.7; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/6; 536/23.7, 24.32; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Searched inventors and keywords: identifying gene growth and library or compar or hybridiz? or nucleic acid or probe array in APS, CAPLUS, MEDLINE, SCISEARCH, BIOSIS WPIDS.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SAPOLSKY et al. Mapping Genomic Library Clones Using Oligonucleotide Arrays. Genomics. May 1996, Vol. 33, No. 3, pages 445-456, see entire document.	1-22
Y	LENNON et al. Hybridization analyses of arrayed cDNA libraries. Trends in Genetics. October 1991, Vol. 7, No. 10, pages 314-317, see entire document.	1-12
Y,P	WO 97/23642 A1 (MICROCIDE PHARMACEUTICALS, INC) 03 July 1997, see abstract and pages 4-7.	1-22
Y,P	WO 97/10365 A1 (AFFYMAX TECHNOLOGIES N.V.) 20 March 1997, see abstract, pages 4-8 and Figure 1.	1-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
12 JANUARY 1998

Date of mailing of the international search report
10 FEB 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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qty:1

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US005591740A

United States Patent [19]

Chipman et al.

[11] Patent Number: 5,591,740

[45] Date f Patent: Jan. 7, 1997

[54] **USE OF DEBROMOHYMENIALDISINE FOR TREATING OSTEOARTHRITIS**

[75] Inventors: **Stewart Chipman**, Reading, Mass.; **David J. Faulkner**, La Jolla, Calif.

[73] Assignees: **OsteoArthritis Sciences, Incorporated**, Cambridge, Mass.; **The Reagents of the University of California**, Oakland, Calif.

[21] Appl. No.: **472,902**

[22] Filed: **Jun. 7, 1995**

[51] **Int. Cl.**[6] **A61K 31/55**

[52] **U.S. Cl.** **514/215**

[58] **Field of Search** 514/215

[56] **References Cited**

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WO93/16703 2/1993 WIPO .

OTHER PUBLICATIONS

Kobayashi, J., et al., "α–Adrenoceptor Blocking Action of Hymenin, A Novel Marine Alkaloid," *Experientia*, 44:86–87 (1988).

Pettit, G. R., et al., "Antineoplastic Agents 168 Isolation and Structure of Axinhydantoin," *Can. J. Chem.*, 68:1621–1624 (1990).

Cimino, S., et al., "Isolation and X–Ray Crystal Struction of a Novel Bromo–Compound from Two Marine Sponges," *Tetrahedron Lett.*, 23(7):767–768 (1982).

Kitagawa, I., "Marine Natural Products. XII. On the Chemical Constituents of the Okinawan Marine Sponge *Hymeniacidon aldis*," *Chem. Pharm. Bull.*, 31(7):2321–2328 (1983).,

Primary Examiner—Theodore J. Criares

Attorney, Agent, or Firm—Hamilton, Brook, Smith & Reynolds, P.C.

[57] **ABSTRACT**

Disclosed is a method of treating osteoarthritis. The method comprises administering a therapeutic amount of debromohymenialdisine to an individual or animal with osteoarthritis. Debromohymenialdisine is able to slow the joint deterioration and cartilage degradation associated with the disease.

12 Claims, 3 Drawing Sheets

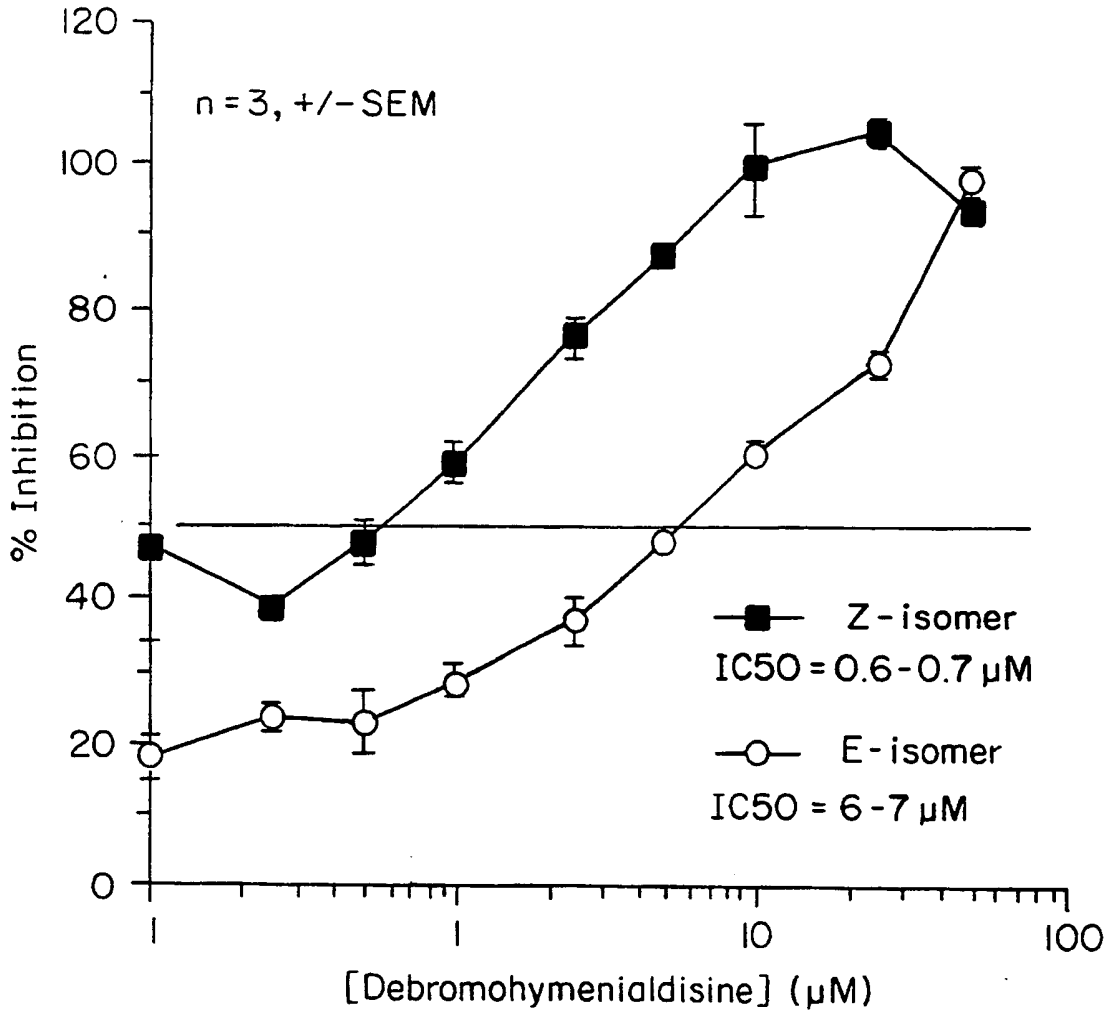

120
100
80
60
40
20
0
% Inhibition
n = 3, +/- SEM
Z - isomer
IC50 = 0.6 - 0.7 μM
E - isomer
IC50 = 6 - 7 μM
1
1
10
100
[Debromohymenialdisine] (μM)

FIG. 1

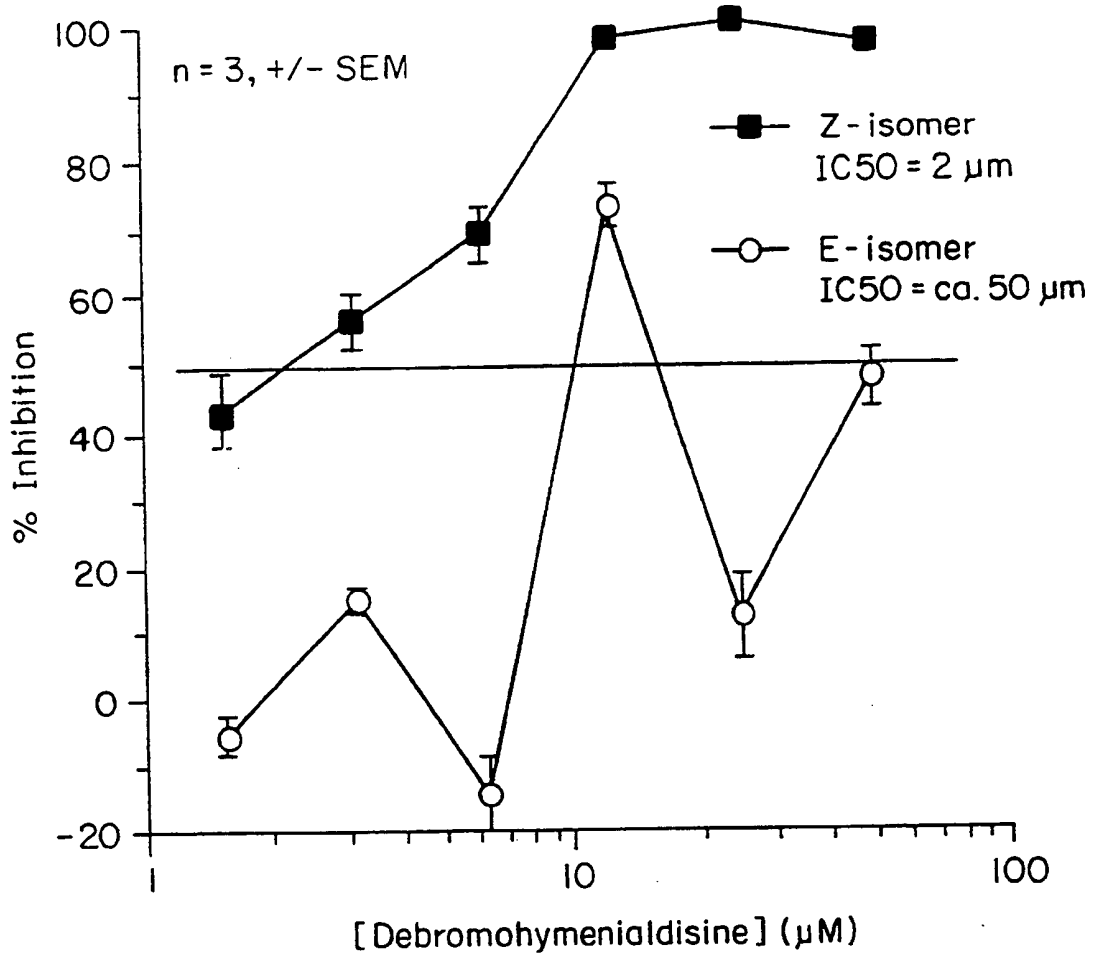

n = 3, +/- SEM
Z-isomer
IC50 = 2 µm
E-isomer
IC50 = ca. 50 µm
% Inhibition
100
80
60
40
20
0
-20
1
10
100
[Debromohymenialdisine] (µM)

FIG. 2

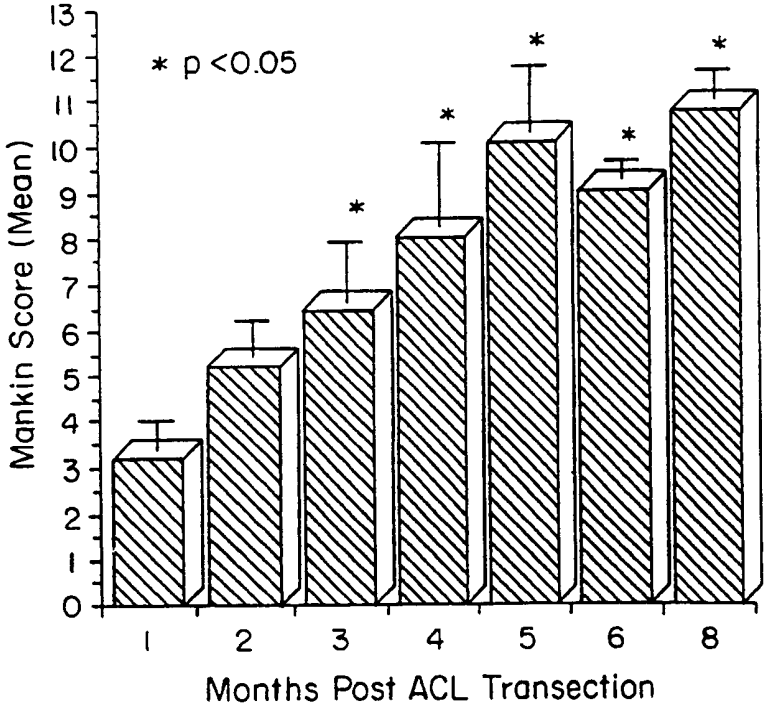
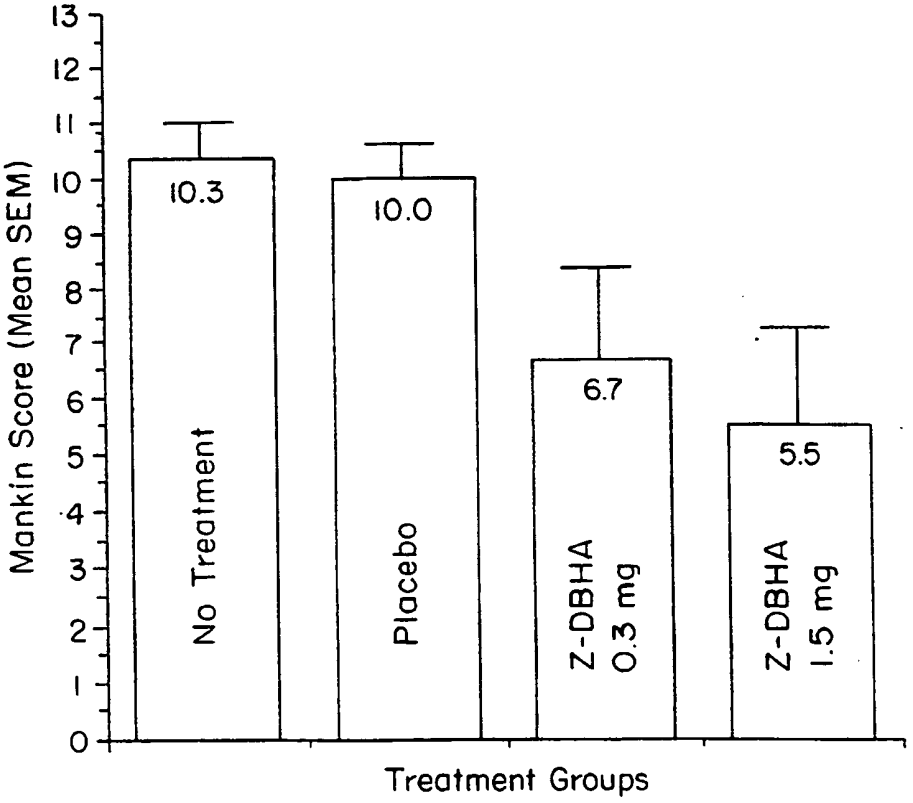

* p <0.05
Mankin Score (Mean)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
1
2
3
4
5
6
8
Months Post ACL Transection

FIG. 3


Mankin Score (Mean SEM)
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1
2
3
4
5
6
7
8
9
10
11
12
13
10.3
No Treatment
10.0
Placebo
6.7
Z-DBHA 0.3 mg
5.5
Z-DBHA 1.5 mg
Treatment Groups

FIG. 4

USE OF DEBROMOHYMENIALDISINE FOR TREATING OSTEOARTHRITIS

BACKGROUND

Osteoarthritis or degenerative joint disease is a slowly progressive, irreversible, often monoarticular disease characterized by pain and loss of function (Mankin and Brandt, Pathogenesis of Osteoarthritis in "Textbook of Rheumatology", Kelly, et al., (eds.) 3rd edition, W. B. Saunders Co., Philadelphia, pp.14699–111471) and Dean, *Arth. Rheum.* 20 (*Suppl.* 2):2 (1991)). The underlying cause of the pain and debilitation is the cartilage degradation that occurs as a result of the disease. A typical end-stage clinical picture includes complete erosion of the weight-bearing articular cartilage, requiring total joint replacement.

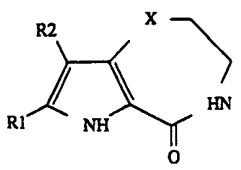
The pro-inflammatory cytokine interleukin-1 (IL-1) plays a major role in the cartilage matrix destructive processes observed in osteoarthritis (Pelletier, et al., *Sem. Arth. Rheum.*, 20:12 (1991) and McDonnell, et al., *Arth. Rheum.*, 35:799 (1992)). IL-1 has been demonstrated to upregulate the synthesis and secretion of the matrix metalloproteinases stromelysin and interstitial collagenase in a dose dependent manner (Stephenson, et al., *Biochem. Biophys. Res. Comm.* 144:583 (1987) and Lefebvre, et al., *Biochem. Biophys. Res. Comm.*, 152:366 (1990). These matrix metalloproteinases are responsible for the damage to the proteoglycan and collagen II components of the cartilage matrix which occur in osteoarthritis (Dean, et al., *J. Clin Invest.*, 84:678 (1989), Mort, et al., *Matrix*, 13:95 (1993) and Buttle, et al., *Arth. Rheum.*, 12:1709 (1993).

Currently, there is no therapeutic approach available that will slow the clinical progression of osteoarthritis, although steroids and non-steroidal anti-inflammatory drugs are used to ameliorate the pain and inflammation associated with the disease. Consequently, there is a need for new therapeutics which slow the joint degeneration caused by osteoarthritis.

SUMMARY OF THE INVENTION

The present invention is based on the discovery that debromohymenialdisine and analogs thereof slow the progression of osteoarthritis. It has also been found that these compounds inhibit the interleukin-1 induced degradation of glycosaminoglycan and extracellular matrix by chondrocytes in culture and explants of articular cartilage. Based on these discoveries, a method of treating osteoarthritis is disclosed.

The method comprises administering to an individual or animal with osteoarthritis a composition comprising a therapeutically effective amount of a compound having the structure of Formula I:

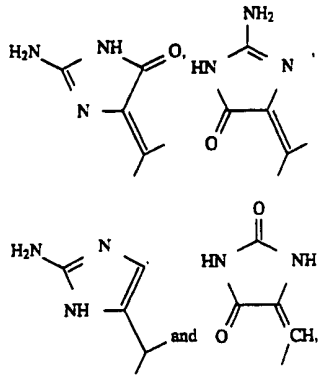


(I)

wherein:

R1 and R2 are each independently selected from the group consisting of -H and a halogen; and

X is selected from the group consisting of:



or physiologically active salts thereof.

The method disclosed herein can slow joint degeneration in individuals with osteoarthritis. As a result, the loss of mobility often suffered by individuals with osteoarthritis can also be slowed. Reduced joint degeneration also results in the individual suffering less pain as a result of the disease.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph illustrating the degree of inhibition of proteoglycan degradation by Z- and E-debromohymenialdisine in the chondrocyte matrix breakdown assay.

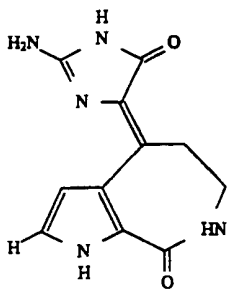
FIG. 2 is a graph illustrating the degree of inhibition of proteoglycan degradation by Z- and E-debromohymenialdisine in the bovine articular cartilage explant assay.

FIG. 3 is a graph illustrating the development of osteoarthritis over time in Hartley guinea pigs following transection of the anterior cruciate ligament.

FIG. 4 is a graph illustrating the effect of Z-debromohymenialdise in slowing the development of osteoarthritis in the Hartley guinea pigs following surgical induction of joint instability.

DETAILED DESCRIPTION OF THE INVENTION

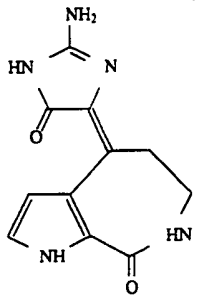
Debromohymenialdisine has been found to be useful in the treatment of osteoarthritis. This compound is a natural product isolated from the marine sponge Hymeniacidon. (Kobayashi, et al., *Experientia.*, 44:86 (1988) and Pettit, et al., *Can. J. Chem.*, 68:1621 (1990)). Both Z -(1) and E- (2) stereoisomers of debromohymenialdisine are known, and can be represented by the structural formulas:



(1)

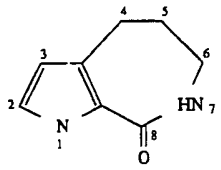
-continued

(2)



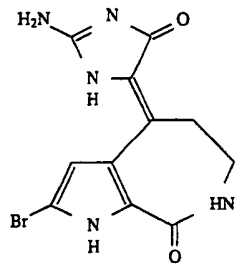
Analogues of debromohymenialdisine are also useful in the treatment of osteoarthritis. These compounds contain the pyrroloazepine ring system found in debromohymenialdisine. This ring system is shown in (3), along with a numbering system for the ring atoms:

(3)

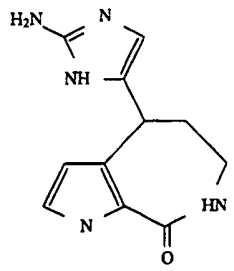


Analogues of debromohymenialdisine also have a five membered, nitrogen-containing heterocyclic ring which is bonded to the four position of the pyrroloazepine ring system. Examples of analogues of debromohymenialdisine include hymenialdisine (4), hymenin (5) and axinohydantoin (6), which have also been isolated from Hymeniacidon and from the marine sponges Axinella and Stylotella and can be represented, as follows:

(4)



(5)



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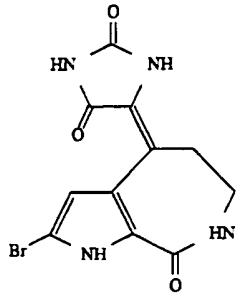
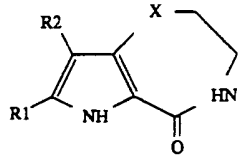
(6)



Compounds used in the method of the present invention include those having the structure shown in Formula II:

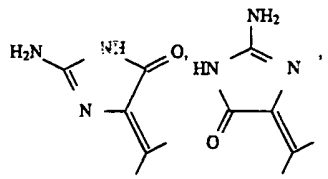
(II)

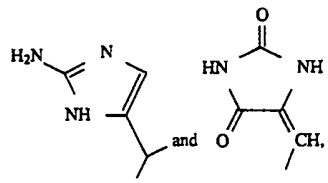


R1 and R2 are each independently chosen from the group consisting of -H and a halogen. Suitable halogens include chlorine, bromine and iodine.

X is CH-A or C=A, wherein A is a five-membered nitrogen-containing heterocyclic ring. Also included are physiologically active salts of the compound.

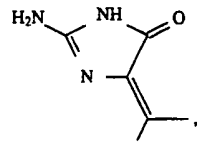
In one embodiment, X is selected from the group consisting of:





In a preferred embodiment X has the structure shown in Formula (IV):

(IV)



and bromine is the preferred halogen.

In a more preferred embodiment R2 is -H. It is most preferred that R1 and R2 are -H, i.e. the compound is Z-debromohymenialdisine.

The method of the present invention can be used to treat individuals, i.e.-humans, or animals wi4th osteoarthritis.

Animals which can be treated with the method include dogs, cats, guinea pigs, farm animals and the like.

A therapeutically effect amount of the compound is the quantity of compound which, after being administered to an individual with osteoarthritis, brings about an amelioration of the disease processes associated with osteoarthritis without causing unacceptable side-effects. "Ameliorating the disease processes associated with osteoarthritis" can include lowering the amount of active matrix metalloproteinase in the individual, e.g. by inhibiting a matrix metalloproteinase, by preventing transcription of a gene which encodes a matrix metalloproteinase, by preventing the synthesis and/or secretion of a matrix metalloproteinase or by preventing interleukin-1 upregulation of matrix metalloproteinase activity. Alternatively, it can also include slowing, arresting or reversing the degradation and loss of function typically observed in a joint afflicted with osteoarthritis.

The skilled artisan will be able to determine the amount of compound which is to be administered to a human or veterinary animal. The amount of compound that is administered to an individual will depend on a number of factors including the general health, size, age, and sex of the individual and the route of administration. It will also depend on the degree, location and severity of the individual's osteoarthritis. One of ordinary skill in the art will be able to determine the precise dosage according to these and other factors. Typically, between about 1 mg per day and about 1000 mg per day are administered to the individual. Preferably, between about 1 mg per day and about 100 mg per day are administered to the individual, more preferably between about 1 mg per day and about 30 mg per day.

The compound can also be administered intraarticularly (for example by injection) into a joint with cartilage degradation caused by osteoarthritis. Other modes of parenteral administration which can be used include systemic administration, such as by intramuscular, intravenous, subcutaneous, or intraperitoneal injection.

In a preferred embodiment, the compound can be administered orally, for example, in capsules, suspensions or tablets. Alternatively, the compound can be administered topically near the joint with cartilage degradation caused by osteoarthritis.

The compound can be administered to the individual in conjunction with an acceptable pharmaceutical carrier as part of a pharmaceutical composition for treating osteoarthritis. Suitable pharmaceutical carriers may contain inert ingredients which do not interact with the compound. Standard pharmaceutical formulation techniques may be employed such as those described in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa. Suitable pharmaceutical carriers for intraarticular and other parenteral administration include, for example, sterile water, physiological saline, bacteriostatic saline (saline containing about 0.9% mg/ml benzyl alcohol), phosphate-buffered saline, Hank's solution, Ringer's-lactate and the like. Methods for encapsulating compositions (such as in a coating of hard gelatin or cyclodextran) are known in the art (Baker, et al., "Controlled Release of Biological Active Agents", John Wiley and Sons, 1986). Suitable carriers for topical administration include commercially available inert gels, liquids supplemented with albumin, methyl cellulose or a collagen matrix. Typical of such formulation are ointments, creams and gels. Preferred carriers for topical administration are those which facilitate penetration of the skin by the compound.

The compound can also be administered as at least one physiologically acceptable salt, such as, the hydrochloride salt, the hydrobromide salt and acetic acid salt.

In another embodiment of the present invention the composition, in addition to the compound, additionally comprises another pharmacologically active agent. Osteoarthritis is characterized by pain in the afflicted joints. Consequently, it is also advantageous to administer the compound with an analgesic or other pain-killing medication. Suitable analgesics include acetyl salicylic acid, acetominophen, and the like.

Osteoarthritis is also characterized by inflammation in the afflicted joints. Consequently, it is also advantageous to administer the compound together with an anti-inflammatory agent such as a non-steroidal anti-inflammatory drug or steroid (e.g. tramcinolone, amcinodide, and the like). Osteoarthritis is also characterized by over-activity of matrix metalloproteinase enzymes. Consequently, it is also advantageous to co-administer the compound with a matrix metalloproteinase inhibitor.

The invention will now be further and specifically described by the following examples.

EXEMPLIFICATION

EXAMPLE 1

Isolation of Z- and E-Debromohymenialdisine Analogs

Debromohymenialdisine was isolated from 250 grams of hydrated Stylotella by successively extracting with 100% methanol (3×1 liter) at room temperature. The combined methanol extracts were concentrated to an aqueous base and partitioned between ethyl acetate. The aqueous phase was concentrated and applied to a C-18 reverse phase HPLC with a water methanol gradient (90:10 to 40:60) as eluent.

Using the chondrocyte matrix breakdown assay described in Example 2, fractions were identified which contained compounds capable of inhibiting interleukin-1 induced cartilage degradation. Preliminary structure determination showed that a class of pyrrole containing compounds were responsible for activity.

Z-Debromohymenialdisine, E-debromohymenialdisine, axinohydantoin and hymenialdisine were isolated from active fractions by further by applying to a C-18 reverse phase HPLC with water:methanol (ratio for the Z-isomer, the E-isomers, axinohydantoin and hymeniadlisine are 82:12, 72:28, 72:28 and 70:30, respectively) and plus the addition of 1% trifluoroacetic acid.

EXAMPLE 2

Inhibition of Cartilage Degradation By Z- and E-Debromohymenialdisine in a Chondrocyte Cell Culture Matrix Breakdown Assay

Isolation of the Cartilage

A cell culture assay was used to-measure the ability of the test compounds to slow the degradation of the extracellular matrix by a metalloproteinase. This assay measured the amount ^{35}S released from chondrocytes grown in a media with ^{35}S labeled sodium sulfate. The cell culture assay was carried out as follows:

Two or three 1 to 3 week old calf joints were obtained from an abattoir. The proximal end of the shank was about 4–5" long to facilitate immobilization in the holder. The joint was kept cool and transported on ice. The exterior of the intact joints was washed well with a suitable anti-microbial

soap, rinsed clean with warm water, rinsed in betadine and then finally rinsed with 70% ethanol. Up to this point all steps were done in a manner to ensure that the joint was kept as clean as possible. All subsequent steps were performed in a sterile field (i.e., in a Edgeguard laminar flow tissue culture hood). The joint was immobilized and the synovial fluid was aspirated with a needle and syringe. The joint was then cut open to expose the articular cartilage using a #21 scalpel. Using locking hemostats, forceps and a #15 scalpel, the cartilage was excised in full thickness pieces. Care was taken not to cut too deep into the subchondral bone to prevent bleeding. The cartilage pieces were placed into a 50 mL centrifuge tube containing 25 mL of Delbecco's phosphate buffered saline (D-PBS) supplemented with 1% antibiotic solution (penicillin, streptomycin and fungizone; GIBCO/BRL). The slices from each joint were then placed into separate 50 mL centrifuge tubes. The D-PBS was decanted and replaced with 25 mL of fresh D-PBS supplemented with antibiotics and subsequently agitated gently.

Enzymatic Digestion

The cartilage pieces were transferred to a fresh 50 mL centrifuge tube and rinsed once more with 25 mL of D-PBS minus antibiotics. An enzymatic digestion solution containing 1 mg/mL of hyaluronidase in serum-free 1:1 DMEM/Ham's F-12 (DMEM/F12) was prepared. This solution was filter sterilized with 0.22 mm Milex—GV filter and kept on ice until ready to use. The cartilage pieces were digested with approximately 5 mL of hyaluronidase solution per joint for 2×15 minutes at 37° C. in the 50 mL centrifuge tube with gentle agitation at the 15 minute mark. This procedure removed residual hyaluronic acid from the surface of the chips. The enzymatic digestion solution was then aspirated and the cartilage pieces were rinsed with 25 mL of D-PBS.

A second enzymatic digestion solution containing 2.5 mg trypsin and 2 mg collagenase P per mL serum-free DMEM/F12 was prepared. This solution was also filter sterilized with a 0.22 mm Millex—GV filter and kept on ice until ready to use. The cartilage pieces were digested with approximately 5 mL of trypsin: collagenase solution per joint for 2×15 minutes at 37° C. in the 50 mL centrifuge tube with gentle agitation at the 15 minute mark. This procedure removed the synovial fibroblasts and any adherent connective tissue from the surface of the chips. The enzymatic digestion solution was then carefully removed and saved and the cartilage pieces were rinsed with 25 mL of D-PBS.

A third enzymatic solution containing 2 mgs of collagenase P (BMB) per mL serum-free DMEM/F12 was prepared. This solution was filter sterilized with a 0.22 mm Millex—GV filter and kept on ice until ready to use. The pre-digested cartilage pieces were finally digested with approximately 20 mL of enzymatic digestion solution per joint for 5–6 hours at 37° C. in a Bellco stirring digestion flask, at which point the cartilage was fully digested away.

Culture and Growth of Isolated Chondrocytes

The enzymes in the synovial fibroblast and chondrocyte digest were neutralized by addition of an equal volume of DMEM/F12 supplemented with 5% fetal bovine serum. Fibroblasts were plated in DMEM at a cellular density of 6.6×10^3 cells per cm^2. The chondrocytes were recovered by filtration through a 70 mm nylon Cell Strainer (Falcon Labware, Inc.), which removed the remaining undigested tissue pieces and clumps of cells. Chondrocytes were then collected by centrifugation at 1000× g for 10 minutes at room temperature. The chondrocytes were then resuspended in 40 mL of DMEM/F12 supplemented with 5% fetal bovine serum. A 200 μL aliquot in 20 mL of isoton was quantitated in a Coulter counter. Chondrocytes were diluted with 1:1 (v/v) DMEM/F-12 supplemented with 5% fetal bovine serum to a density of 2×10^4 cells per cm^2 of culture surface. This density allowed the cells to be at confluence as soon as they are plated. Four days later the cells were again fed with media. This time period ensured the attachment of the chondrocytes to the plastic well.

Chondrocytes were plated at 8×10^4 cells/2 cm^2 per well with 0.5 mL of 1:1 (v/v) DMEM/F12 supplemented with 10% fetal bovine serum in 24 well plates and incubated for 4 days. The cultures were then fed on days 4, 7, 11, 14, 18 and 21 with 0.5 mL/well of DMEM/F12 plus 10% fetal bovine serum. At this time the cells were densely confluent and have developed a three-dimensional extracellular matrix.

Radiolabel & Chase of Chondrocytes

On day 22, the wells are rinsed 2×1 mL with D-PBS and incubated for 30 minutes in 0.5 mL of DMEM/F12 per well. This starve media was removed, replaced with 0.5 mL/well of DMEM/F23 plus 10 μCi ^{35}S labeled sodium sulfate per well and incubated for 48 hours at 37° C. On day 24, the labeling media is removed. The wells were then re-fed with 0.5 mL of DMEM/F12 plus 10% fetal bovine serum. The cultures were "chased" with cold sulfate (in the tissue culture media) for two more days and on day 26 were re-fed with 0.5 mL of fresh DMEM/F12 plus 10% fetal bovine serum.

Experimental Addition and Harvest

On day 27, the wells were rinsed 2×1 mL with D-PBS and incubated for 22–24 hours with 0.5 mL/well of serum free DMEM/F12, 1 ng/ml of rhIL-1α, plus the compound being tested at the desired concentrations. The wells were carefully rinsed to remove any residual fetal bovine serum which could affect the final results. A first control was run in which the assay was carried out in the absence of the compound being tested. A second control was also run in which the assay was carried out in the absence of test compound and rhIL-1α. On day 28 the 0.5 mL of media was removed and counted in a mini-vial with 4 mL of scintillation fluid. The cell layer was rinsed 1×1 mL with D-PBS and harvested with 0.5 mL of 1× trypsin-EDTA (purchased from Gibco-BRC, Life Technologies, Gaithersburg, Md.) (incubated for at least 15–20 minutes) for scintillation counting as before. The data is expressed as percent radiolabel released in the media of the total according to the formula:

$$\% \text{ release} = \frac{cpm_{media}}{(cpm_{media}) + (cpm_{cell\ layer})}$$

The average percent release is used to determine a percent inhibition according to the following formula:

$$\% \text{ Inhibition} = \frac{A-B}{C-B} \times 100,$$

wherein,
A=% release in presence of test compound;
B=% release in control; and
C=% release in presence of rhIL-1α.

Z-debromohymenialdisine, E-debromohymenialdisine, hymenialdisine and axinohydantoin were tested in the chondrocyte matrix breakdown assay described above. The IC_{50} value for each compound tested in the assay is given in the Table below. Z-debromohymenialdisine and E-

TABLE

Inhibition of Proteoglycan Degradation by Marine Alkaloids from Stylotella

Compound	IC_{50}
Z-debromohymemialdisine (1)	0.8 μM
E-debromohymemialdisine	.6 μM
Axinohydantoin (3)	>50 μM
Hymenialdisine (2)	>50 μM

debromohymenialdisine both inhibited proteoglycan degradation. The Z- isomer was more active than the E-isomer with IC_{50}s of 0.8 and 6 μM, respectively. FIG. 1 show the inhibition of proteoglycan degradation by the Z-and E-isomer of debromohymenialdisine over a range of concentrations. The data are expressed as percent inhibition compared to control and interleukin-1 stimulated cultures versus inhibitor concentration. Each data point represents an average of four wells plus or minus the standard error of the mean. Both compounds exhibited a broad, shallow dose response curve.

Example 3

Inhibition of Cartilage Degradation in the Bovine Cartilage

Explant Assay by Z- and E-Debromohymenialdisine

A tissue culture assay was used to measure the ability of the compounds of the present invention to slow the degradation of the extracellular matrix by metalloproteinases. This assay measured the amount of ^{35}S-glycosaminoglycan (^{35}S-GAG) released from labeled bovine cartilage explants.

Knee joints from a 1 to 3 week old calf were obtained immediately after sacrifice from the Abattoir and then transported on ice. The intact joints were washed well with tap water and soaked in 50% (v/v) Povidine iodine solution, obtained from Burre National, Inc., Baltimore, Md. All subsequent steps were performed in a laminar flow tissue culture hood using standard sterile technique. The joint was immobilized in a shank holder and the joint capsule was cut open to expose the articular cartilage. Cartilage explant plugs, approximately 15 mg wet weight, were removed from the flat articulating surfaces in the lower-most region of the knee joint using a sterile steel cork-borer and collected in a 250 mL roller bottle containing about 100 mL fresh Delbecco's minimum essential medium (DMEM), obtained from Gibco BRC, Life Technologies, Gaithersburg, Md., containing 4.5 g/l (D)-glucose and (L)-glutamine, without sodium pyruvate. The fresh media also contained enough Hepes buffer and sodium bicarbonate such that the pH was about 7.4. The media was then further supplemented just before use with 100 units Penicillin, 100 μg Streptomycin, and 50 μg (L)-ascorbic acid per mL of medium.

Once collected, the explant plugs were washed f ur times with 50 mL fresh DMEM. The plugs were then placed in the incubator for a minimum of 1 hour to equilibrate, before proceeding to make disks from the articulating surface of each plug. A 1 mm thick disk was sliced from individual plugs from the end that was the articulating surface of the joint. The plug was held steady in the sterile template (4 mm diameter ×1.5 mm deep) using sterile forceps. A scalpel blade was used to carefully slice off the disk. Only the superficial articulating surface responded well in culture.

Individual disks obtained were transferred to a tissue culture flask containing about 100 mL fresh media. The flask containing the disks was placed in an incubator at 37° C. (with 5% CO_2, 95% air) and allowed to equilibrate overnight and at least one additional day before labeling. When ready to label, the old media was replaced with 50 mL fresh media containing about 500 μCi ^{35}S-Sodium Sulfate. The plugs were labeled in bulk for about 48 hours. The next morning, the "hot" media was removed and replaced with fresh "cold" media. The disks were again allowed to equilibrate overnight before being used for actual experiments.

The media in which the disks were stored was changed immediately prior to performing the assay. The disks were then returned to the incubator until the test media and the two control media had been prepared. The test media consisted of the desired concentration of a compound being tested for its ability to inhibit extracellular matrix degradation and concomitant recombinant human Interleukin rhIL-1α (5 ng/mL) in fresh DMEM solution. The control media were identical to the test media, except that the first control media lacked rhIL-1α and the second control media lacked the test compound. 250 μL of each of the test and control media were transferred to separate 96-well TC plates. Flamed forceps were used to transfer a disk from the incubator to each 96-well TC plates that had been filled with either the test media or one of the two control media.

The TC plates were then placed in the incubator and cultured for 3–4 days (initial incubation with rhIL-1α alpha takes at least 3 days to stimulate endogenous metalloproteinases). A 50 μL aliquot of media from each TC plate was saved and counted. The rest of the media was removed with a suction device.

The cartilage disks from each TC plate were also saved for counting. The disks were removed with forceps and placed in microcentrifuge tubes and then dissolved in 250 μL of full strength Formic Acid. The tubes were capped and placed at 65°–70° C. in a block-heater for 4–6 hours. A 50 μL aliquot was then counted.

The percent ^{35}S-GAG release is calculated as follows:

$$\%\ ^{35}S\text{-}GAG\ \text{release} = \{(cpm_{medium})/(cpm_{medium}+cpm_{explant})\} \times 100\%$$

The percent inhibition at 50 μM of extracellular matrix damage in cartilage explant was calculated as follows:

$$\%\ \text{Inhibition} = \frac{(A-B)-(C-B)}{(A-B)} \times 100,$$

wherein

A=% GAG release induced by rhIL-1α;

B=% GAG release in the absence of rhIL-1α; and

C=% GAG release in the presence of rhIL-1α plus 50 μM of compound being tested.

Both Z-debromohymenialdisine and E-debromohymenialdisine were tested in the bovine cartilage explant assay, as described above. Both the Z- and E-isomers inhibited interleukin-1 stimulated proteoglycan degradation with IC_{50}s of 2 μM and 50 μM, respectively. FIG. 2 sh ws the percent inhibition of these tw compounds over a range f concentrations compared with control and interleukin-1 stimulated cultures versus inhibitor concentration. Each data

point represents an average of four wells plus or minus the standard error of the mean. Both compounds exhibited a broad, shallow dose response curve in both assay systems.

EXAMPLE 4

Induction of Osteoarthritis in Guinea Pigs by Surgical

Induction of Joint Instability

Thirty five male Hartley guinea pigs 40 days old, were purchased from Charles River Laboratories. Each group consisted of 5 animals that were euthanized at 1, 2, 3, 4, 5, 6 and 8 months post-surgery. Ketamine 40 mg/kg, Xylazine 5 mg/kg and Fentanyl 0.1 mg/kg sc were used for anesthesia. Naloxone hydrochloride was given at 0.1 mg/kg following surgery. For radiography, animals were sedated with Xylazine 10 mg/kg sc and superficially anesthetized with Ketamine 20 mg/kg sc.

Surgery was performed under direct visualization using a dissecting microscope at 7.5 fold magnification. A medial longitudinal parapatellar incision was followed by division of the capsule and synovial layers. A partial medical menisectomy was followed by transection of the anterior cruciate ligament. Confirmation of the ligament transection was obtained by a positive anterior drawer sign. The capsule was continuously sutured with 5-0 VICRYL™ and the skin was closed with staples.

After the animals were sacrificed, joints were prepared for histological examination by standard techniques. Joints were fixed for 14 days in 10% formalin. Knees were then decalcified in 20% Formic acid in PBS, pH 7.2 for 24 hours. Sections cut at 5μ were stained with Safranin O, Fast Green and Hematoxylin. Radiographic evaluation was performed with antero-posterior and lateral views. A dental X-Ray unit and DF-50 film were used. Histopathological scoring was performed by two blinded, independent observers using the Mankin criteria (Mankin, H. J., "Biochemical and Metabolic Aspects of Osteoarthritis," *Orthopedic Clinics of North America*, 2:19–30 (1971). This evaluation system grades the structural defects, cellular changes, loss of proteoglycan as reflected by Safranin 0, and disruption of an antatomic landmark reflecting the transition to calcified cartilage. The Mankin scoring criteria are listed below:

Mankin Scoring Criteria	
Structure	
Normal	0
Surface Irregularities	1
Pannus and Surface Irregularities	2
Clefts to the Transition Zone	3
Clefts to the Calcified Zone	4
Complete Disorganization	5
Cells	
Normal	0
Diffuse Hypercellularity	1
Cloning	2
Hypocellularity	3
Safranin O Straining	
Normal	0
Slight Reduction	1
Moderate Reduction	2
Severe Reduction	3
No dye noted	4

-continued

Mankin Scoring Criteria	
Tidemark Integrity	
Intact	0
Cross by Blood Vessels	1

Lesions were initially localized to the weight bearing regions on the medial side of the tibia plateau and femoral condyle. The developing osteoarthritis was characterized by cartilage erosion and fibrillation. Radiographic examination revealed sclerosis, cysts and osteophytes. Osteophytes were first noted at three months post-surgery.

The histopathological evaluation of the knee by the Mankin Criteria is depicted in the FIG. 3. The results indicate that there was disease progression starting from a Mean Mankin score of 3.2 (±1.3) at one month post-surgery to 10.7 (±0.7) at 8 months. Changes were statistically significant ($p<0.05$) when compared with the group at 1 month. Changes observed included cartilage hypertrophy, erosion and fibrillation, loss of proteoglycan, chondrocyte cloning and osteophytes. The Hartley guinea pig has been previously reported to have a high incidence of spontaneous osteoarthritic disease (Bendele, A. M. and Julman, J. F. "Spontaneous Cartilage Degeneration in Guinea Pigs," *Arthritis and Rheumatism*, 31:561–565 (1988). The surgical procedure presented here augments this propensity. The fidelity and anatomic location of the lesions presented and the progressive nature of the disease indicates that Hartley guinea pigs which have undergone the procedure described above are a valid model for drug evaluation studies for the study of osteoarthritis.

EXAMPLE 5

Slowing the Development of Osteoarthritis by

Dehybromohymenialdisine (Z-DBHA) in the Hartley Guinea Pig

Model

Surgery was performed on twenty-four male Hartley guinea pigs during which the joint instability was induced according to the procedure described in Example 4.

The animals were divided into four groups of six. Beginning two weeks following surgery, each animal in the first two groups was given weekly intraarticular injections into the surgically treated joints of 0.3 mg (6 mg/ml) or 1.5 mg of Z-debromohymenialdisine (30 mg/ml) in a vehicle containing carboxymethylcellulose (2 mg/ml saline) and polysorbate 80 (0.5 mg/ml saline). Total volume of the injection was 50 μl. Group III animals were given weekly intraarticular injections of a placebo, while Group IV animals were left untreated. The treatments were continued for seven weeks.

At the termination of the system, the animals were euthanized and radiographic evaluation of the knees was performed as described in Example 5 using the Mankin criteria. The results of the histological evaluation at seven weeks post surgery are depicted in FIG. 4. Results for each f the Mankin criteria are given in the Table.

TABLE

	Structure	Cells	Safranin O	Tidemark
No Treatment	4.6	1.6	2.3	4/6
Z-DBHA 0.3 mg	3.5	1.2	1.5	3/6
Z-DBHA 1.5 mg	1.6	1.5	1.1	1/6

Value given for Structure, Cells, and Safranin O are mean values.

Values for Tidemark Penetration are incidence figures.

The results indicate that there was a disease progression to a mean Mankin score of 10.3 for untreated animals and 10.0 for animals treated with the placebo. In contrast, animals treated with Z-debromohymenialdisine showed a significant reduction in disease progression. A dose of 0.3 mg/week resulted in disease progression to a mean Mankin score of 6.7, while a dose of 1.5 mg/week resulted in disease progression to a mean Mankin score of only 5.5. The results with 1.5 mg/week dose regimen, when compared to the No Treatment group by an unpaired t test gave a p value of 0.03 (significant) while the lower dose regiment of 0.3 mg/week gave a p value of 0.07 (marginally significant). When compared by non-parametric tests, which makes no assumption about the distribution of the data, the high dose (1.5 mg/week) was marginally significant (p=0.06) and the lower dose (0.3 mg/week) had a value compared to the No Treatment group of 0.130 (not significant).

In all cases every index of the Mankin scoring criteria has shown improvement when compared with the No Treatment Group. In all but the cellular evaluation the higher dose (1.5 mg/week) gave values that were consistent with a dose responsive relationship. Thus, Z-debromohymenialdisine was able to diminish the structural and biochemical changes as they are reflected by the loss of Safranin O staining in developing osteoarthritis.

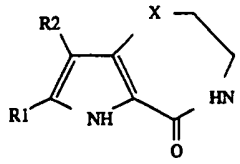
These results demonstrate that Z-debromohymenialdisine is effective in slowing joint degeneration and cartilage degradation associated with osteoarthritis in animal models.

Equivalents

Those skilled in the art will know, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

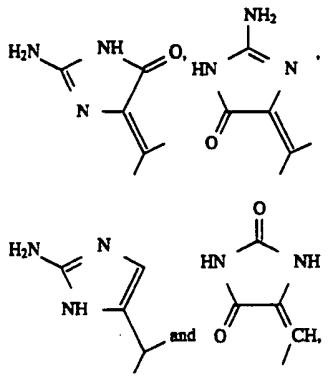
We claim:

1. A method of treating an individual or animal with osteoarthritis, comprising administering to the individual a composition comprising a therapeutically effective amount of a compound having the following structure:



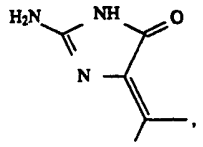
wherein R1 and R2 are each independently selected from the group consisting of -H and a halogen; and

wherein X is selected from the group consisting of:



and physiologically active salts thereof.

2. The method of claim 1 wherein X is:



and physiologically active salts thereof.

3. The method of claim 2 wherein the halogen is -Br.

4. The method of claim 2 wherein R1 is H.

5. The method of claim 2 wherein R1 and R2 are -H.

6. The method of claim 5 wherein the composition is administered orally.

7. The method of claim 5 wherein the composition is administered topically.

8. The method of claim 5 wherein the composition is administered parenterally.

9. The method of claim 5 wherein the composition is administered intra-articularly.

10. The method of claim 5 wherein the composition additionally comprises a pharmacologically active agent.

11. The method of claim 10 wherein the pharmacologically active agent is selected from the group consisting of an analgesic, asteroid, a non-steroidal anti-inflammatory drug and a matrix metalloproteinase inhibitor.

12. The method of claim 5 wherein the composition additionally comprises a suitable pharmaceutical carrier.

* * * * *